Biophysical Chemistry, 46 (1993) 1–11
Elsevier Science Publishers B.V., Amsterdam

BIOCHE 01713

Local anesthetic–phospholipid interactions. Effects of ionic strength, temperature, and phospholipid mixtures on the binding of dibucaine to phospholipids

Samira A. Barghouthi [1], Ravinder K. Puri [2] and Maurice R. Eftink

Department of Chemistry, University of Mississippi, University, MS 38677 (USA)

(Received 19 May 1992, accepted 5 August 1992)

Abstract

The nature of the interaction of amphipathic drugs, such as dibucaine, with phospholipid bilayer membranes was investigated using equilibrium dialysis. Profiles for the binding of cationic dibucaine to unilamellar vesicles were obtained at different temperature and ionic strengths, and for mixtures of neutral phospholipid dimyristylphosphatidylcholine (DMPC) with negatively charged dimyristylphosphatidylglycerol (DMPG). The degree of binding of the cationic drug at pH 5 was found to be higher at temperatures above the T_m of DMPC (24°C) than below T_m. Also enhanced drug binding was found to occur as the concentration of monovalent salt was increased (0.01–0.85 M) and as the percentage of DMPG was increased. Using the Stern and Guoy–Chapman model, which takes into consideration electrostatic effects, we were able to simultaneously fit all our binding data with a minimum of fitting parameters. These parameters (for data at 45°C) are an association constant, K, of 330 M^{-1}, a maximum possible number of drug molecules absorbed per unit surface of vesicle, σ_+^m, of 1.70×10^{-2} Å^2, and a surface area per bound drug, γ_D, of 48 Å^2. The data were fitted equally well by an alternate model in which binding of the drug is described as a partitioning equilibrium, with factors included for electrostatic effects and surface expansion caused by drug intercalation between the fatty acid chains.

Keywords: Dibucaine; Phospholipid vesicles; Electrostatic effects; Guoy–Chapman–Stern model

1. Introduction

One of the sites of action of local anesthetics is thought to be phospholipid membranes [1]. The

Correspondence to: M.R. Eftink, Department of Chemistry, University of Mississippi, University, MS 38677 (USA).

[1] Present address, Department of Chemistry and Physics, Southeastern Louisiana State University, Hammond, LA 70402 (USA).

[2] Present address, Environmental Trace Substance Research Center, Columbia, MO 65203 (USA).

Abbreviations used: DMPC, L-α-dimyristylphosphatidylcholine; DMPG, L-α-dimyristylphosphatidylglycerol; γ_D, molecular surface area of bound drug in a vesicle bilayer in Å^2; γ_L, molecular area of a phospholipid in a bilayer in Å^2; K_{Cl}, association constant of chloride to bound dibucaine; K_L, association constant of dibucaine to vesicles according to saturable model, in units of M^{-1}; K_p, partition coefficient of dibucaine into vesicles according to non-saturable model, in units of M^{-1} Å^{-2}; ν, moles of bound drug per mole of phospholipid; σ_+ and σ_-, surface charge density (per Å^2) due to bound cationic drug and due to anionic phospholipid, respectively; σ_+^{max}, maximum surface charge density at saturation with bound dibucaine; Ψ_0, surface potential; T_m, gel-to-liquid crystalline phase transition temperature; X_{DMPG}, mole fraction of DMPG in vesicle.

incorporation of local anesthetics, and other amphipathic molecules, into membranes causes changes in the thermodynamic properties of the latter. The surface charge of biological membranes will be altered by the adsorption of drugs, if they are charged, hence affecting the surface potential. Also, the binding of drugs can perturb the phase transition temperature, T_m, of bilayer systems (i.e., pure phospholipid vesicles); this effect may be due to an intercalation of the drug between fatty acid changes (thus interfering with the cooperative unit) or to a preferential interaction with the polar head groups of either the gel or liquid crystalline phase.

A variety of biophysical approaches have been taken to study the thermodynamic and structural aspects of the interaction of amphipathic drugs with model membranes. Among these methods include various magnetic resonance studies [2–6], fluorescence spectroscopy [7–9], differential scanning calorimetry [10,11], film balance measurements [12–18], microelectrophoresis [15], and a variety of procedures for determining equilibrium binding isotherms (i.e., gel filtration chromatography [16], equilibrium dialysis [17], centrifugation [18], fluorescence [19] and difference spectroscopy [20]). When the binding of drug has been compared for biomembrane preparations and model phospholipid vesicles, most studies have demonstrated similar binding behavior for the drug [21,22].

Here we report thermodynamic studies of the binding of the cationic form of the local anesthetic, dibucaine (2-butoxy-*N*[2-(diethylamino)-ethyl]-4-quinoline carboxamide), to unilamellar phosphatidylcholine vesicles. Dibucaine (see Fig. 1) is selected for this study because, firstly, it is known to perturb the T_m of phosphatidylcholine bilayers [23,24], and, secondly, dibucaine is fluorescent, which allows for handy determination of its relative concentration [26]. Previously we studied the binding of dibucaine to phosphatidylcholine vesicles as a function of pH [17]. In the present study we present results of the binding of dibucaine as a function of temperature, ionic strength, and the mole fraction of phosphatidylglycerol (which introduces a negature surface charge to vesicles). We use equilibrium dialysis to obtain binding isotherm data; this simple method avoids potential complications of methods such as centrifugation (e.g., trapping of excess drug) and spectral methods (e.g., linearity of the spectral change with the degree of binding). We employ a pH (= 5) that is much below the pK_a (= 8.9) of dibucaine, to ensure that the data are for binding the mono-cationic form of the drug. We will show that electrostatic effects dominate the interaction, even with neutral vesicles. The Guoy–Chapman relationship will be employed to describe these electrostatic effects and we will show that this model can be simultaneously fitted to multiple sets of data (e.g., binding isotherms at different salt concentration). Finally, we will compare models in which the binding of the cationic drugs is considered to reach saturation and in which saturation is not included.

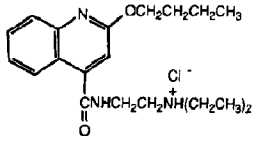

Fig. 1. Structure of dibucaine-hydrochloride.

The primary purpose of this study is to collect binding profiles, to study the role of electrostatic effects in binding, and to attempt to distinguish between binding models. A secondary purpose is to establish conditions where most of dibucaine is membrane bound for the steady-state and time-resolved fluorescence studies in the following paper [7].

2. Experimental section

L-α-Dimyristylphosphatidylcholine (DMPC) and L-α-dimyristylphosphatidylglycerol (DMPG), dibucaine-HCl, and Fiske–Subbarow reducing agents were obtained from Sigma Chemical Co. Single lamellar phospholipid vesicles were prepared using the procedure of Barenholz and coworkers [27]. The lipid phosphate concentration was determined using the method of Bartlett [28].

A 16-cell equilibrium dialysis chamber, together with Spectra-por 3 dialysis sheets, was used for the binding studies following procedures of Eftink and coworkers [17]. All experiments were done at pH 5 (in sodium acetate of 0.05 *M*, unless mentioned otherwise). We loaded the equilibrium dialysis chambers with varying concentrations of dibucaine solutions (16×10^{-3} *M* to 1.0×10^{-4} *M*) on one side and phospholipid (approximately 1×10^{-2} *M*) on the other side. The dialysis chamber was incubated in a shaking water bath at 45°C (unless specified otherwise) for at least 12 hours. We then added aliquots (25 μl), from each side of the dialysis cells, to 2.0 ml of 0.05 *M* acetate buffer. The fluorescence intensity was then measured at 390 nm with excitation at 340 nm, using a Perkin-Elmer MPF 44A spectrofluorometer and was compared to standards of known concentration [17]. This provided the molar concentration of dibucaine on both sides of the dialysis chamber (free dibucaine, $[D]_f$, on one side and free plus bound, $[D]_f + [D]_b$, on the lipid side). The moles of drug bound per mole of phospholipid, ν, was then calculated as $[D]_b$/[Lipid], for various values of $[D]_f$. A check was made that the phospholipid does not diffuse across the dialysis membrane during the 12 hour equilibration time. This was done by attempting to measure lipid phosphate on the opposite chamber as a function of time; no diffusion of phospholipid across the dialysis membrane was observed during this time period.

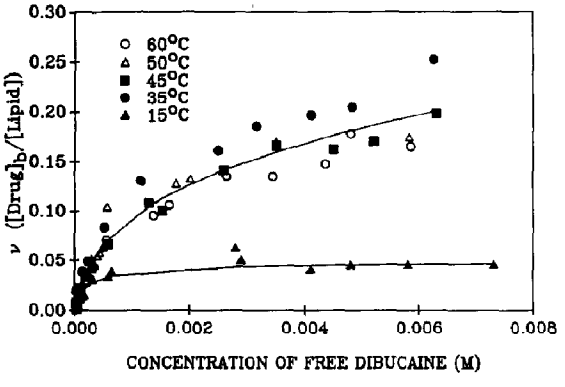

Fig. 2. Binding profiles for the association of dibucaine to DMPC unilamellar vesicles at 15°C (▲), 35°C (●), 45°C (■), 50°C (△) and 60°C (○). Conditions; ionic strength of 0.05 *M* and approximately 1×10^{-2} DMPC.

Equilibrium dialysis experiments were performed at different temperatures (15–60°C), ionic strength (0.01–0.85 *M*), and for various DMPG/DMPC phospholipid mixtures (0–30% DMPG). We have obtained saturation isotherms which show a degree of negative cooperativity. Various models (see following section) were fitted to these data using the non-linear least squares program NONLIN [29]. The analysis was performed simultaneously over several related data sets, as will be explained in the following sections.

3. Results and discussion

3.1 Binding isotherms

From equilibrium dialysis experiments we obtained binding isotherms of ν (moles of bound drug per mole of phospholipid) versus the concentration of free dibucaine $[D]_f$. Figures 2–5 represent binding profiles for dibucaine to DMPC as a function of temperature, ionic strength, and percentage of added DMPG. In all cases, Scatchard plots (e.g., Fig. 3B) show that the cationic dibucaine binds to phospholipids with an apparent negative cooperativity. As shown in Fig. 2, the binding of dibucaine to DMPC model membranes at 0.05 M ionic strength approaches saturation at ν of 0.2–0.3 (i.e., 0.2–0.3 drug molecules bound per one phospholipid) at temperatures above the T_m for DMPC vesicles ($T_m \approx$ 24°C). At temperatures below T_m, we found that dibucaine still binds to DMPC and approaches saturation at ν of about 0.04.

The binding of dibucaine is enhanced upon increasing the ionic strength as shown in Fig. 3 (data obtained at 45°C). The maximum number of drug molecules bound per phospholipid appears to almost double upon changing the ionic strength from 0.05 to 0.25 M. Binding isotherms at ionic strength ranging from 0.01 to 0.85 are shown in Fig. 4.

In Fig. 5 is shown the effect of changing the charge density of phospholipid on the binding of the cationic drug (at 45°C). Here DMPG, with a

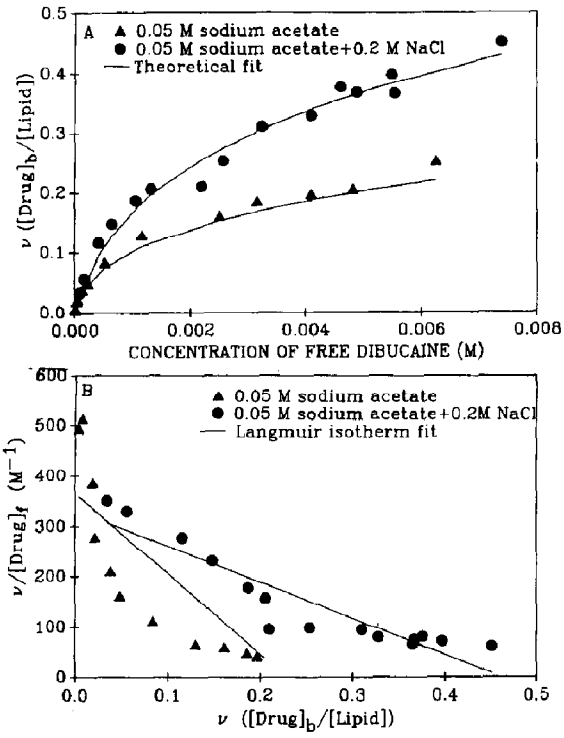

Fig. 3. (A) Binding profiles for the association of dibucaine to DMPC unilamellar vesicles at ionic strength of 0.05 *M* sodium acetate (▲), and 0.05 *M* sodium acetate +0.2 *M* NaCl (●). The solid lines are a fit to the Gouy–Chapman–Stern model as described in the text. Conditions; 45°C, approximately 1×10^{-2} *M* DMPC. (B) Scatchard plots of the above data. The solid lines are forced first of the Scatchard equation.

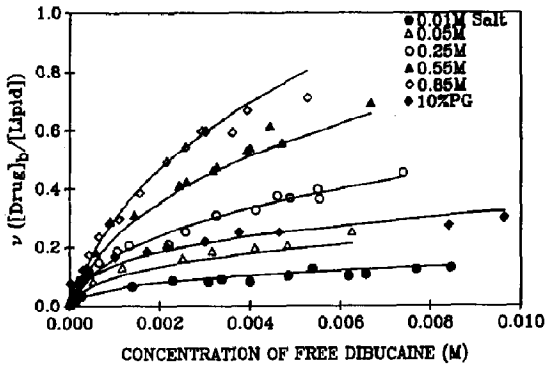

Fig. 4. Binding profiles for the association of dibucaine to DMPC and to DMPC/DMPG unilamellar vesicles at 0.01 *M* acetate (●), 0.05 *M* acetate (△), 0.05 *M* acetate +0.2 *M* sodium chloride (○), 0.05 *M* acetate +0.5 *M* sodium chloride (▲), 0.05 *M* acetate +0.8 *M* sodium chloride (◇) and 10% PG in 0.05 *M* acetate (♦). The solid lines are a simultaneous fit to the Gouy–Chapman–Stern model as described in the text. Conditions; 45°C, approximately 1×10^{-2} *M* DMPC.

net negative charge, was introduced into the vesicles. We found that the maximum degree of binding was increased (i.e., saturation approached at ~0.5 drugs per phospholipid at 30% DMPG, as compared to saturation of ~0.25 drug per phospholipid in 100% DMPC). From these ionic strength and DMPG studies it is clear that the binding of dibucaine is influenced by electrostatic interactions. As illustrated by Figure 3B, analysis of these binding isotherms in terms of the Scatchard equation (i.e., a Langmuir type isotherm, $\nu = nK[D]/(1+K[D])$, where K is an association constant, n is the number of binding sites per phospholipid, and [D] is free drug concentration) is unsatisfactory; forced-fits give n and K values that depend on ionic strength and the percentage of DMPG. Below we present models which directly include the electrostatic interactions.

3.2 Binding models

The above binding isotherms clearly indicate that electrostatic interactions play a dominant role in the association of dibucaine with phospholipid bilayers. Here we present two models for

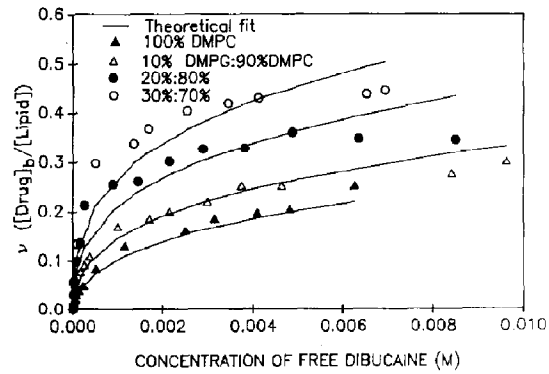

Fig. 5. Binding profiles for the association of dibucaine to DMPC/DMPG unilamellar vesicles with 0% DMPG (▲), 10% DMPG (△), 20% DMPG (●) and 30% DMPG (○). The solid lines are a fit to the Gouy–Chapman–Stern model as described in the text. Conditions; 45°C, approximately 1×10^{-2} *M* DMPC.

analyzing these data. These models, which employ Guoy–Chapman theory to estimate the surface charge effects, have been presented by several researchers [20,24,30–33]. The difference between the models is that one assumes saturable binding (Langmuir isotherm) of the drug to the vesicle, whereas the other model assumes nonsaturable binding (Henry's law phase partitioning). We will show that the binding data in Figs. 4 and 5 can be described by both models, provided the models take into account the expansion of the bilayers upon drug intercalation and the binding of chloride counterions, as well as the electrostatic interactions. Our theoretical treatments are not new, but we show here the strategy of simultaneously analyzing multiple data sets (i.e., data as a function of ionic strength) in order to test the binding models.

We will first present in detail the saturable binding (Langmuir) model; the unsaturable binding (partitioning) model is simply a truncation of the former and will be presented more briefly.

3.2.1 Saturable binding (Langmuir) model

This and the following model describe the electrostatic interactions at the bilayer surface in a non-specific manner using the Guoy–Chapman expression for the relationship between the charge density, σ, on a bilayer and its surface potential, Ψ_0 [34]. The surface charge density on a vesicle can be due, in our system, to either (a) the reversible binding of cationic dibucaine (contributing a positive charge density, σ_-, which is related to the amount of bound drug) or (b) the presence of anionic DMPG in the bilayer (contributing a negative charge density, σ_+, which is related to the mole fraction of DMPG, X_{DMPG}). These two sources of surface charge density (charge per Å^2) lead to repulsive and attractive, respectively, electrostatic interactions with an approaching cationic dibucaine molecule. These interactions can be related to the membrane surface potential, Ψ_0, by the following (Grahame) approximation of the Guoy–Chapman relationship

$$\exp(-F\Psi_0/RT) = \left(1+\sigma_+^2/4\varepsilon\varepsilon_0 RTc\right)^{-1} \quad (1a)$$

$$\exp(-F\Psi_0/RT) = \left(1+\sigma_-^2/4\varepsilon\varepsilon_0 RTc\right) \quad (1b)$$

where ε is the dielectric constant of water, ε_0 is the permittivity of space, R is the gas constant, F is the Faraday constant, and c is the total concentration of monovalent salt in solution.

Whether Ψ_0 is produced by reversible binding of cationic drug or the presence of anionic DMPG, the local (at the vesicle surface) concentration of the cationic drug $(DH^+)_0$ will be determined by the Boltzmann relationship

$$(DH^+)_0 = (DH^+)_\infty \exp(-F\Psi_0/RT) \quad (2)$$

where $(DH^+)_\infty$ is the concentration of cationic drug at an infinite distance from the bilayer surface

In the saturable model, a Langmuir isotherm is assumed for the binding process, with the drug concentration being that at the membrane surface.

$$\nu = \frac{nK_L[DH^+]_0}{1+K_L[DH^+]_0} \quad (3)$$

Here ν is the moles of bound drug per mole of phospholipid, K_L is the association constant, and n is the maximum number of drug binding sites per phospholipid. If the drug binds only in its cationic form, then the value of ν is related to σ_+ as $\nu = \sigma_+ \gamma_L$, where γ_L is the surface area (in Å^2) per phospholipid molecule. (See below for the inclusion of surface expansion effects). Since ν is related to σ_+, the Langmuir isotherm can also be expressed in terms of charge density

$$\sigma_+ = \frac{\sigma_+^{\max} K_L[DH^+]_0}{1+K_L[DH^+]_0} \quad (4)$$

where $\sigma_+^{\max}$ is the maximum surface charge density (due to reversible drug binding).

The Guoy–Chapman, Boltzmann, and Langmuir isotherm equations can be combined to include electrostatic effects in drug binding. First, we consider only the repulsive interaction for the binding of cationic drug (to an initially neutral bilayer surface). Combining eqs. (1a) and (2) yields

$$[DH^+]_0 = [DH^+]_\infty/\left(1+\sigma_+^2/Ac\right) \quad (5)$$

where $A = 4\varepsilon\varepsilon_0 RT$ ($= 1.46\times10^{-5}$ at 45°C). On

substitution of this expression for $[DH^+]_0$ into eq. (4), we obtain

$$\sigma_+ = \nu/\gamma_L = \frac{\sigma_+^{max} K_L [DH^+]_\infty}{1 + \sigma_+^2/Ac + K_L[DH^+]_\infty} \quad (6)$$

Extension of this model to include the attractive interaction with anionic DMPG is achieved by factoring in eq. (1b) to yield.

$$[DH^+]_0 = \frac{[DH^+]_\infty (1 + \sigma_-^2/Ac)}{(1 + \sigma_+^2/Ac)} \quad (7)$$

and

$$\sigma_+ = \nu/\gamma_L = \frac{\sigma_+^{max} K_L [DH^+]_\infty (1 + \sigma_-^2/Ac)}{1 + \sigma_+^2/Ac + K_L[DH^+]_\infty (1 + \sigma_-^2/Ac)} \quad (8)$$

This equation, which has been called the Guoy–Chapman–Stern relationship [30], describes ν to be a function of K_L, σ_+^{max}, γ_L, and σ_-. The latter two parameters will be known with some certainty; γ_L has been determined from film balance measurements [35] and σ_- is calculated as $\sigma_- = X_{DMPC}/\gamma_L$, where X_{DMPG} is the mole fraction of phospholipid that is anionic. This equation could be used to fit the binding isotherm data in Figs. 4 and 5, but it can be further modified to include corrections for (a) the expansion of the bilayers due to the intercalation of drug, and (b) the weak, reversible binding of anionic species.

If the drug binds by intercalating, for which there is evidence for dibucaine [14], then expansion of the bilayer will result and this will diminish the repulsion between like charges. A correction for expansion can be made by the following expression for the effective surface area of the bilayer

$$\gamma_L' = \gamma_L + \gamma_D \nu \quad (9a)$$

$$= \gamma_L (1 + \nu \gamma_D/\gamma_L) \quad (9b)$$

where γ_L and γ_D are the surface areas of a phospholipid and of bound drug in a monolayer.

An additional consideration is that the association (or dissociation) of a specific ion (including hydrogen ion) will neutralize either the bound drug or DMPG. Since the pK_a of bound dibucaine is estimated to be about 8 [31] and since the experimental pH (= 5) is well below this, the binding of neutral dibucaine can be ignored in this study. However, elevated chloride concentrations are employed and one can consider the weak association of Cl^- with bound dibucaine [25]. This would neutralize the latter and would enable more drug to bind without producing a repulsive positive charge density. A thermodynamic scheme for the co-binding of chloride counterions is given below

$$\begin{array}{ccc} D^+ + Cl^- & \rightleftharpoons & D \cdot Cl \\ \Big\| K\,(DMPC) & & \Big\| K\,(DMPC) \\ DMPC \cdot D^+ + Cl^- & \overset{K_{Cl}}{\rightleftharpoons} & DMPC \cdot D \cdot Cl \end{array}$$

where K_{Cl} is defined as $[DMPC \cdot D \cdot Cl]/[DMPC \cdot D^+][Cl^-]$, i.e., the association of Cl^- to bound dibucaine.

The ν value can then be considered to include both cationic and neutral drug forms,

$$\nu = \frac{[D^+]_{bound} + [DCl]_{bound}}{[lipid]} \quad (10a)$$

$$= \nu_{D+} + \nu_{DCl} \quad (10b)$$

$$= \nu_{D+}(1 + K_{Cl}[Cl^-]) \quad (10c)$$

Combining the binding isotherm (eq. 8) with the corrections for surface expansion and for the binding of chloride counterions, we obtain the following relationship for the total binding of drug

$$\nu = \sigma_+ \gamma_L (1 + \nu \gamma_D/\gamma_L)(1 + K_{Cl}[Cl^-]) \quad (11)$$

$$\nu = \frac{\sigma_+^{max} K_L [DH^+]_\infty \gamma_L \left(1 + \frac{\sigma_-^2}{Ac}\right)\left(1 + \nu \frac{\gamma_D}{\gamma_L}\right)(1 + K_{Cl}[Cl^-])}{\left(1 + \frac{\sigma_+^2}{Ac}\right) + K_L[DH^+]_\infty \left(1 + \frac{\sigma_-^2}{Ac}\right)} \quad (12)$$

$$= \frac{\sigma_+^{max} K_L [DH^+]_\infty \gamma_L (\text{elec attract})(\text{expansion corr})(\text{chloride binding corr})}{(\text{elec repulsion}) + K_L[DH^+]_\infty (\text{elec attract})} \quad (13)$$

This transcendental equation contains the following adjustable parameters: σ_+^{max}, K_L, K_{Cl}, γ_D and γ_L (the latter parameter may be estimated by independent Langmuir–Blodgett surface area/pressure measurements). The value of σ_- is de-

termined as $\sigma_- = X_{DMPG}/\gamma'_L$, and σ_+ is determined as $\sigma_+ = \nu/(\gamma'_L(1 + K_{Cl}[Cl]))$. Thus eq. (12) contains the unknown ν on both sides. To fit this function to experimental ν vs. $[DH^+]_\infty$ data, we have written a program that employs a Newton–Raphson iterative approximation routine together with a nonlinear least squares analysis (NONLIN [29]). Also, since eq. (12) is a function of both the monovalent salt concentration, c, and mole fraction of DMPG, X_{DMPG}, we have written this program to simultaneously fit eq. (12) to multiple data sets (i.e., at different c and/or X_{DMPG}).

3.2.2 Unsaturable binding (partitioning) model

The above model assumes that there is a maximum number of binding sites per phospholipid (or a maximum charge density, σ_+^{max}). Seelig and coworkers [20] and Westman and coworkers [32,33] have argued that, while a saturable binding model may be appropriate for surface binding ligands [35], amphipathic drugs dibucaine may more correctly be considered to bind in an unsaturable manner. Film balance experiments show that dibucaine penetrates, at least partially, between fatty acid chains and causes an expansion of phospholipid monolayers [14]. The above researchers argue further that such intercalation and expansion should not be a saturable process. They have presented, instead, a partitioning model to interpret drug–membrane binding data. This model substitutes the following Henry's law partitioning isotherm for the Langmuir adsorption isotherm

$$\nu = K_p[DH^+]_0 \tag{14}$$

where K_p is a partition coefficient for drug between the membrane and aqueous phases. At first glance, it does not appear that the above partitioning isotherm can account for the data in Figs. 2–5 which appear to approach saturation. That is, the above function is for a linear relationship between ν and drug concentration. However, inclusion of the term for repulsive electrostatic interaction, between bound dibucaine molecules, causes this isotherm to also have a "saturating" appearance. Inclusion of the electrostatic terms, plus the factors for surface expansion and the binding of chloride counterions, yields the following partitioning function.

$$\nu = \frac{K_p[DH^+]_\infty \gamma_L(1 + \sigma_-^2/Ac)(1 + \nu\gamma_D/\gamma_L)(1 + K_{Cl}[Cl^-])}{(1 + \sigma_+^2/Ac)} \tag{15}$$

Table 1

Fitting parameters for the analysis of binding isotherm data [a] by the saturating (Langmuir) model and the non-saturating (Henry's) model

Data sets	K_L (M^{-1})	γ_D (Å^2)	K_{Cl} (M^{-1})	σ_+^{max} (e/Å^2)	SSR
Langmuir isotherm model					
Five salt concentrations	3.33×10^2 (3.06–3.66)	48.6 (39.2–57.7)	0.323 (0.254–0.394)	1.71×10^{-2} (1.58–1.83)	2.96×10^{-2}
Five salt and 10% DMPG	3.26×10^2 (3.00–3.60)	46.9 (36.5–56.4)	0.456 (0.353–0.568)	1.54×10^{-2} (1.40–1.64)	4.76×10^{-2}
	K_p (M^{-1} Å^{-2})	γ_D (Å^2)	K_{Cl} (M^{-1})		SSR
Henry's isotherm model					
Five salt concentrations	5.43 (4.93–5.92)	30.0 (22.5–36.9)	0.321 (0.235–0.414)		2.88×10^{-2}
Five salt and 10% DMPG	4.55 (4.11–4.98)	25.6 (15.5–34.6)	0.512 (0.378–0.661)		5.22×10^{-2}

[a] All data obtained at pH 5.0, 45°C. The five salt concentrations were 0.01 M, 0.05 M, 0.25 M, 0.55 M, and 0.85 M. The SSR values are the sum of the squares of the residuals for a non-linear least squares analysis (simultaneous over the indicated data sets). The values in parentheses are 67% confidence limits for the indicated parameters.

This differs from eq. (12) only in the absence of σ_+^{max}, the absence of a term in the denominator, and the definition of K_p (vs. K_L). K_p is defined by the above equation to be a partition coefficient per unit surface area of the bilayer and has units of M^{-1} Å^{-2}. We have also written a computer program to fit this partitioning function to data, including the ability to simultaneously fit multiple data sets.

3.3 Analysis of data

The binding data in Fig. 4 were fitted by both the saturating (Langmuir) model and the unsaturating (Henry's) model. The results of these analyses are listed in Table 1. Simultaneous non-linear least squares analyses were preformed over the following multiple data sets; (a) the isotherm data at five salt concentrations form 0.01 to 0.85 M, and (b) the same five salt concentration data plus the isotherm data for 10% DMPG containing vesicles (the latter at an ionic strength of 0.05 M). In Table 1 are listed the fitting parameters for the two models and for the two sets of binding isotherms; also listed are the 67% confidence limits for the fitting parameters and the sum of the squares of the residues (SSR) as an indicator of the goodness of the fits.

The saturating (Langmuir) model has four fitting parameters, K_L, γ_D, σ_+^{max}, and K_{Cl}. The values of K_L and σ_+^{max} are very highly correlated and the first listed in Table 1 were achieved by alternately fixing K_L or σ_+^{max} and floating the other three parameters. Inclusion of the 10% DMPG data diminished slightly the quality of the fit (compare the SSR values). However, the theoretical curves in Fig. 4 show a very satisfactory simultaneous fitting of the six isotherms (including the 10% DMPG data) to the Langmuir based isotherms (eq. 12).

The recovered K_L value of $\sim 3.3 \times 10^2$ M^{-1} (at 45°C) indicates that there is a modest affinity of dibucaine for the phospholipid vesicle, in the absence of electrostatic effects. The value of σ_+^{max} is approximately equal to the reciprocal of the surface area of a phospholipid molecule ($1/\gamma_L = 1.4 \times 10^{-2}$ Å^{-2}), suggesting that, at saturation, there will be approximately one dibucaine per phospholipid molecule. The recovered value of the surface area for bound dibucaine, γ_D, is ~ 48 Å^2, a value which is reasonable in comparison with the value of $\gamma_L = 70$ Å^2 taken for a phospholipid molecule. Finally, the value of $K_{Cl} \approx 0.3$ to 0.5 M^{-1} is in the range expected for the interaction of monovalent ions with a charged phospholipid surface [31].

The non-saturating (Henry's) isotherm model yields fits with nearly the same SSR and requires only three fitting parameters, K_p, γ_D, and K_{Cl}. The fits listed in Table 1 were made by floating all three parameters. The value of $K_p = 5$ M^{-1} Å^{-2} may seem small, however, this is defined as a value per unit surface area. By multiplying K_p by γ_L (70 Å^2) we obtain an effective K_p' value of ≈ 350 M^{-1}, which is more in line with values previously found for the partition coefficient of dibucaine into phospholipid vesicles (see ref. [15] and [20] and note the different pH and tempera-

Table 2

Fitting parameters for analysis of binding isotherms by the non-saturating (Henry's) model [a]

Data sets	K_p (M^{-1} Å^2)	γ_D (Å^2)	K_{Cl} (M^{-1})	SSR
Five salt concentrations	5.43 (4.93–5.92)	30.0 (22.5–36.9)	0.321 (0.235–0.414)	2.88×10^{-2}
Five salt concentrations	4.69 (4.04–4.71)	⟨70⟩	0.092 (0.039–0.144)	6.38×10^{-2}
Five salt concentrations	5.66 (4.55–6.16)	⟨0⟩	0.686 (0.402–0.790)	4.80×10^{-2}
Five salt concentrations	6.33 (5.69–6.95)	50.8 (43.4–58.3)	⟨0⟩	5.84×10^{-2}

[a] All data obtained at pH 5.0, 45°C. The values in angle brackets (⟨ ⟩) were fixed in the analysis.

ture used in our study). The γ_D value is found to be 25–30 Å^2 and K_{Cl} is found to be 0.3–0.6 M^{-1} according to this model.

We also performed analyses in which certain of the parameters were fixed in order to demonstrate their importance in the fits. In Table 2 are listed the results of the fitting of the "five salt" data set to the non-saturating model for cases in which (a) γ_D was fixed at 70 Å^2 (i.e., at the same value as the phospholipid), (b) γ_D was fixed at 0 Å^2 (i.e., for the case in which there is no surface expansion on drug binding), and (c) K_{Cl} was fixed at 0 M^{-1} (i.e., no counterion binding). In each of these cases, the fits are significantly worse, as indicated by the higher SSR values.

3.4 Molecular interpretations

The results and fits raise the following set of questions. Do the binding isotherms reflect binding to both the inside and outside monolayers of the vesicles? Does dibucaine induce a micellization of the vesicles? Does expansion occur when dibucaine binds to the vesicles? To what extent does dibucaine intercalate between the fatty acids chains (or does binding occur only at the phosphorylcholine head region)? Is the binding process saturable or not?

Apparent values of ν are found as high as 0.7 at high salt concentration (see Fig. 4). The calculation of ν is based on total phospholipid molecules, whether they are on the internal or external monolayer of the vesicle. In a small unilamellar vesicle, approximately two-thirds of the DMPC molecules are initially on the external monolayer [36,37]. If binding only occurs on the external monolayer, the actual ν values would be $\sim 3/2$ times the apparent values. This would mean that the ratio of bound drug to external DMPC molecules would be as high as 0.5 to 1.0 under many conditions. If dibucaine does intercalate and cause monolayer expansion, as our fitting and the work of others [14,20,32,33] indicates (see below), then association *only* on the outer monolayer would seem to be impossible, since this would require that the outer monolayer would expand by up to 100% without an obvious mechanism for expansion of the inner monolayer. Binding of dibucaine to both the inner and outer monolayers would seem more reasonable, since it would avoid this problem.

The question then is whether dibucaine, at pH 5, can permeate the bilayer on our time scale for equilibrium dialysis. NMR studies with vesicle entrapped paramagnetic shift-ions have been interpreted as showing evidence for the rapid transverse diffusion of a similar local anesthetic, tetracaine [38]. Conflicting data of this sort and interpretation were later reported by Eriksson [39]. We show in the following article that dibucaine, added to the outside of vesicles, can rapidly (with minutes) quench the fluorescence of a fatty acid probe incorporated into both internal and external monolayers. Mayer et al. [40] used a rapid gel filtration method to monitor the kinetics of the accumulation of cationic dibucaine into PC vesicles in response to the presence of a transmembrane potential (negative inside). The half-life for this uptake was found to be about 10 min. In the absence of the transmembrane potential a smaller accumulation of dibucaine occurred and the half-life for this non-driven uptake was on the order of 10–60 min. Based on these studies we conclude that dibucaine binds to both the inner and outer monolayers of a unilamellar vesicle.

The results of Mayer et al. [40] are also consistent with transmembrane co-diffusion of a dibucaine $H^+ \cdots Cl^-$ ion-pair being a route of accumulation of the drug in vesicles. In the present work we have no direct evidence to support this conclusion. However, the co-diffusion of such an ion pair would avoid the generation of a transmembrane potential or pH gradient.

The question of whether dibucaine causes a micellization of PC vesicles was addressed in the work of Eriksson [39]. Based on ^{31}P NMR studies of the phosphorylcholine head groups, he found that micellization of egg PC vesicles only occurs above 69 mM dibucaine. Our equilibrium binding studies were performed at a much lower drug concentration. Thus there is no evidence that the vesicular state of the phospholipids has been altered.

The Langmuir film balance studies of Seelig [14] have shown that the association of cationic dibucaine with PC monolayers causes an expan-

sion of the membrane surface area. This, of course, is most easily interpreted in terms of the intercalation of dibucaine between the fatty acid chains of the PC molecules. Seelig concluded that the bound, intercalated dibucaine molecules have an apparent surface are of $\gamma_D = 55$ Å^2, as compared to a value of $\gamma_L = 62$ Å^2 for the PC used. With the saturating or non-saturating binding models, our data in Fig. 4 are best fit with values of $\gamma_D \approx 48$ Å^2 and 30 Å^2, respectively. Either γ_D value is reasonable, in comparison to the molecular dimensions of a phospholipid molecule, for intercalation to occur. Spectroscopic data are consistent with some penetration of dibucaine into phospholipid bilayers, but the actual degree of penetration is not clear. Eriksson [39] showed that the binding of dibucaine causes a progressive change in the ^{1}H NMR chemical shift of the *N*-methyl choline resonance, suggesting a perturbation of the conformation of the phosphoryl head group. Fluorescence studies show that the emission of dibucaine is blue shifted and protected from quenching by iodide upon binding to phospholipids [7,13]. These results suggest that the quinoline ring is at least partially buried in the bilayer. Time-resolved anisotropy decay studies (see following article) show a decrease in the rotational motion of bound dibucaine. Assuming that the tertiary ammonium group of bound dibucaine lies at the aqueous interface and/or interacts with the negatively charge phosphate groups of phospholipids, it appears that the butoxyquinoline group wedges its way into the hydrophobic region, and that there is slow rotational motion of the quinoline ring.

The question of whether the binding process is saturating or non-saturating may not be possible to answer unequivocally. Due to the obviously downward curving binding curves (i.e. Figs. 2–5), quantitative analyses of the binding of amphipathic drugs to membranes have assumed a Langmuir (saturation) binding function with the apparent negative cooperativity being due to electrostatic effects [17,30–33]. As pointed out by Seelig et al. [20], a partitioning (non-saturating) model can also predict downward curving binding profiles, when electrostatic interactions are included. We are able to fit the data in Fig. 4 almost equally well with both models. (The data as a function of X_{DMPG} in Fig. 5 is not fitted as well by either model. This is easily attributed to the fact that, for any DMPG composition, the percentage of the DMPG on the outer monolayer will be larger than that on the inner monolayer [41].) Since the partitioning model contains one less fitting parameter, it must be considered the more acceptable model, provided that it is physically reasonable and consistent with other data. Since there is separate evidence that binding of dibucaine involves partial penetration and surface expansion [14], it seems reasonable that binding would behave as a non-saturable process. Only at very high drug concentration would this by difficult to rationalize; it would imply that a bilayer membrane structures would persist even when the number of bound drug is greater than the number of phospholipid molecules.

Regardless of the question of saturating versus non-saturating binding, electrostatic interactions are shown to play a dominant role in determining the extent of drug binding. The intrinsic association constant of dibucaine for the bilayers is a modest value of $K_L = 3 \times 10^2\ M^{-1}$ (or $K_p' = 3.5 \times 10^2\ M^{-1}$). Electrostatic repulsion, between bound cationic dibucaine molecules, attenuate binding. Electrostatic attraction to anionic DMPG enhances binding, but the models are consistent with this being a non-specific (Stern-layer) effect, rather than a change in the intrinsic association constant. The poorer binding of dibucaine to the gel state of DMPC also appears to be primarily a consequence of there being a greater surface charge density (due to the smaller γ_L of the gel state) caused by cationic drug binding to this state. Finally, we observe that, for the liquid crystalline phase, the binding of dibucaine shows a slight decrease in affinity with increasing temperature (see Fig. 2). These data are consistent with a recent determination by Seelig and Ganz [42] that the enthalpy change for the binding of dibucaine is small and exothermic.

Acknowledgements

We thank Dr. Glen Ramsay for assistance with the data analysis, and we thank Dr. Michael

Johnson, University of Virginia, for kindly providing a copy of NONLIN. This research was supported by NSF Grant DMB 91-06377.

References

1 D. Papahadjopoulos, Biochim. Biophys. Acta 265 (1972) 169–186.
2 J.L. Browning and H. Akutsu, Biochim. Biophys. Acta 684 (1982) 172–178.
3 E.C. Kelusky and I.C.P. Smith, Biochemistry 22 (1983) 6011–6017.
4 Y. Boulanger, S. Schreier and I.C.P. Smith, Biochemistry 20 (1981) 6824–6830.
5 Y. Kuroda and Y. Fujiwara, Biochim. Biophys. Acta 903 (1987) 395–410.
6 H.H. Wang, J. Earnest and H.P. Limbacher, Proc. Natl. Acad. Sci. USA 80 (1983) 5297–5301.
7 S. Barghouthi and M.R. Eftink, Biophys. Chem. 46 (1993) 13–19.
8 W.K. Surewicz and W. Leyko, Biochim. Biophys. Acta 643 (1981) 387–397.
9 T.G. Burke and T.R. Tritton, Biochemistry 24 (1985) 1768 1776.
10 D. Papahadjopoulos, K. Jacobson, G. Poste and G. Shepherd, Biochim. Biophys. Acta, 394 (1975) 504–519.
11 Gutierrez-Merino, C., Molina, A., Escudero, B., Diez, A. and Laynez, J. (1989) Biochemistry 28, 3398–3406.
12 Beurer, G. and Galla, H.J., (1987) Eur. Biophys. J., 14, 403–408.
13 T. Guilmin, E. Goormaghtigh, R. Brasseur, J. Caspers and J.M. Ruysschaert, Biochim. Biophys. Acta 605 (1982) 169–176.
14 A. Seelig, Biochim. Biophys. Acta 899 (1987) 196–204.
15 S. Ohki, Biochim. Biophys. Acta 777 (1984) 56–66.
16 C. Huang and J.P. Charlton, Biochemistry 11 (1972) 735–740.
17 M.R. Eftink, R.K. Puri and M.D. Ghahramani, Biochim. Biophys. Acta 813 (1985) 137–140.
18 M.J. Conrad and S.J. Singer, Biochemistry 20 (1981) 808–818.
19 P. Wooley, Biophys. Chem. 10 (1979) 289–303.
20 A. Seelig, P.R. Allegrini and J. Seelig, Biochim. Biophys. Acta 939 (1988) 267–276.
21 W.J. Pjura, A.M. Kleinfeld and M.J. Rovanovsky, Biochemistry 23 (1984) 2039–2043.
22 M. Luxant and H. Galla, Biochim. Biophys. Acta. 856 (1986) 274–282.
23 A.G. Lee, Biochim. Biophys. Acta 448 (1976) 34–44.
24 A.G. Lee, Biochim. Biophys. Acta 514 (1978) 95–104.
25 Y. Kaminoh, H. Kamaya and I. Ueda, Biochim. Biophys. Acta 987 (1989) 63–68.
26 G. Vanderkooi, Photochem Photobiol. 39 (1984) 755–762.
27 Y. Barenholz, D. Gibbes, B.J. Litman, J. Goll, T.E. Thompson and F.D. Carlson, Biochemistry 16 (1977) 2806–2810.
28 G.R. Bartlett, J. Biol. Chem. 234 (1959) 466–468.
29 M.L. Johnson and S.G. Frasier, Methods Enzymol. 117 (1985) 301–342.
30 S. McLaughlin and H. Harary, Biochemistry 15 (1976) 1941–1948.
31 E.K. Roony and A.G. Lee, Biochim. Biophys. Acta. 732 (1983) 428–440.
32 J. Westman, L.E.G. Eriksson and A. Ehrenberg, Biophys. Chem. 19 (1984) 57–68.
33 J. Westman, Y. Boulanger, A. Ehrenberg, I.C.P. Smith, Biochim. Biophys. Acta 685 (1982) 315–328.
34 S. McLaughlin, Annu. Rev. Biophys. Chem. 18 (1989) 113–136.
35 C. Altenbach and J. Seelig, Biochim. Biophys. Acta 818 (1985) 410–415.
36 J.-P. Dufour, R. Nunnally, L. Buhle, Jr. and T.Y. Tsong, Biochemistry 20 (1981) 5576–5586.
37 A. Tardieu, V. Luzzati and F.C. Reman, J. Mol. Biol. 75 (1973) 711–733.
38 P. Schlieper and L. Michelis, Biophys. Struct. Mech. 10 (1983) 1–8.
39 L.E.G. Eriksson, Biophys. Chem. 26 (1987) 9–18.
40 L.D. Mayer, M.B. Bally, M.J. Hope and P.R. Callis, J. Biol. Chem. 260 (1985) 802–808.
41 D.M. Michaelson, A.G. Horwitz and M.P. Klein, Biochemistry 12 (1973) 2637–2645.
42 J. Seelig and P. Gauz, Biochemistry 30 (1991) 9354–9359.